Cereal powdery mildew effectors: a complex toolbox for an obligate pathogen

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Abstract: Highlights • Cereal powdery mildews have evolved a large repertoire of candidate effector proteins. • These effectors are encoded within size variable and highly diversified gene families. • Mildew effectors can act as virulence factors, Avr genes and Avr suppressors. • Their mode of action provides a molecular basis for the Zig-Zag evolutionary model.

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Cereal powdery mildew effectors: a complex toolbox for an obligate pathogen

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Cereal powdery mildews are major pathogens of cultivated monocot crops, and all are obligate biotrophic fungi that can only grow and reproduce on living hosts. This lifestyle is combined with extreme host specialization where every mildew subspecies (referred to as *forma specialis*) can only infect one plant species. Recently there has been much progress in our understanding of the possible roles effectors play in this complex host–pathogen interaction. Here, we review current knowledge on the origin, evolution, and mode of action of cereal mildew effectors, with a particular focus on recent advances in the identification of *bona fide* effectors and avirulence effector proteins from wheat and barley powdery mildews.

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Introduction

Powdery mildews are agronomically important fungal pathogens infecting a wide range of monocot and dicot crops. Cereal powdery mildew diseases are caused by only one species, *Blumeria graminis*, which can be divided into several subspecies, corresponding to highly specialized pathogens infecting only one specific crop species. These are sometimes referred to as '*forma specialis*' (f.sp., literally forms belonging to one host species). For instance, *Blumeria graminis tritici* (*B.g.* f. sp. *tritici*), *B.g. hordei*, and *B.g. secalis*, can only grow on wheat, barley, or rye respectively [1••,2,3]. All cereal mildews are obligate biotrophs, meaning that they can only grow and reproduce on living host tissue. Considering the fact that this lifestyle is combined with extreme host specialization, it was proposed that highly complex and yet poorly understood mechanisms regulate host–pathogen interactions in cereal mildews [4••,5••]. Whole genome sequencing of the wheat and barley powdery mildews revealed on the one hand a drastic reduction of the gene content compared to other ascomycete pathogens, and on the other hand an expansion of the putative effector gene complement [4••,5••]. In this context, we propose that cereal powdery mildews provide a highly informative system to study the molecular role of effectors in pathogen virulence and host adaptation, based on a case of extreme host specialization. This review aims at providing a concise overview of current knowledge in effector biology of cereal mildews. In particular, we summarise advances in effector gene identification and functional characterization. Finally, possible ways for accelerating effector gene function discovery in cereal mildews will be discussed.

Genome-wide identification of candidate effectors in cereal mildew genomes

In powdery mildews, putative effectors have been identified using whole genome sequencing, and large-scale analysis of the fungal proteome, based on *a priori* criteria defining groups of protein coding genes that eventually constituted the putative effectors. Thus, Candidate Secreted Effector Proteins (CSEPs) from wheat and barley powdery mildew were defined as predicted secreted proteins (i.e. including an N-terminal 'signal peptide') that did not have trans-membrane domains, and did not have homologues in non-mildew fungi [4••,5••]. Based on these criteria, initial sets of 472 and 437 CSEPs were identified in the barley and wheat powdery mildew genomes, respectively. There have been continuous efforts to improve effector gene prediction in mildews which has resulted in the identification of larger sets of 722 and 734 CSEPs in the barley and wheat powdery mildew genomes, respectively [6••,7].

In addition to genome information, powdery mildew effectors were also mined in the proteome of *B.g. hordei*, by identifying proteins that are found in fractions enriched with isolated haustoria [8] or in infected barley epidermis devoid of epiphytic fungal material [9,10]. In the latter work, to generate a group of proteins named Blumeria Effector Candidate (BEC) proteins, the criterion of excluding homologs present in non-mildew fungi

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was not used. This has led to the identification of effector proteins such as BEC1005 and BEC1019, which are necessary for full virulence in barley powdery mildew, and are broadly conserved in ascomycete fungi [11,12]. Interestingly, there is nearly complete overlap between the set of barley powdery mildew CSEPs and the BECs; that is, there are only five BECs which are not included in the CSEP set [13].

Origin and evolution of mildew effectors

The Erysiphales, that is the fungi causing powdery mildews, are an ancient monophyletic group which are estimated to have originated within the Leotiomycetes over 120 million years ago (Mya) [14]. The closest sister group is the non-pathogenic Myxotrichaceae, while all extant Erysiphales are obligate biotrophic pathogens of plants. It is reasonable to assume that effector proteins played essential roles in the early evolution of the powdery mildew fungi. One good example are the genes encoding for effector proteins with a predicted fungal ribonuclease-like (RNase-like) three dimensional structure similar to the ribonuclease T1 from *Aspergillus phoenicis* [6••]. The genes encoding the so-called RNase-like effectors have a single intron in a strictly conserved position [15]. Remarkably, both RNase-like effectors and the conserved intron are present in *Erysiphe necator* the agent of grapevine powdery mildew, which is thought to have diverged from the cereal mildew fungi in the late Cretaceous, over 70 Mya [Spanu and Dry, unpublished results]. Therefore, it is likely that this class of mildew effectors accounting for ca. 10–15% of all predicted mildew CSEPs and represented in six out of the 20 largest mildew effector families [6••,7] derived from a single ancestor similar to the canonical fungal RNase T1 [16].

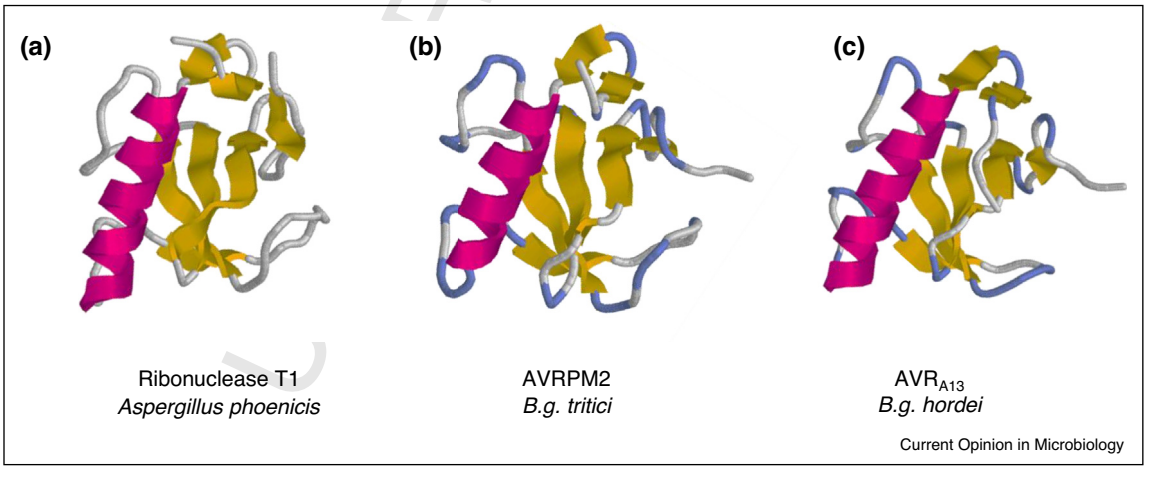
In a recent study, Menardo and colleagues found a new class of CSEPs with structural homologies with the MD2-related lipid-recognition (ML) domain [7], which is predicted to be involved in binding to specific lipids (IPR003172) [17]. Notably, the gene family encoding for ML-like CSEPs is conserved in distinctly different lineages of grass powdery mildews, hinting this novel class of mildew effectors may also be derived from a common ancestor [7].

Cereal powdery mildew effector families are under stronger diversifying selection than non-effector genes, and show extreme levels of sequence variation and gene turnover [7], while the predicted structures appear conserved (Figures 1 and 2a) [18]. Essentially, only the protein sequence encoding the predicted signal peptide is highly conserved among members of the same family. Immediately after the putative cleavage site, sequence homology is reduced to a conserved variant of the YxC motif [6••], and the position of a few amino acids including residues of putative structural importance such as cysteines and prolines (Figure 2a). This high level of sequence divergence might be a result of a strong diversifying selection pressure imposed by the host immune system. Indeed, several RNase-like effectors from wheat, barley, and rye powdery mildews interact with nucleotide-binding leucine-rich repeat (NLR) immune receptors from the host, or suppress such a recognition, suggesting they are likely under selection to evolve [19••,20,21••,22••].

Mode of action of cereal mildew effectors

The first insights into the mode of action of cereal mildew effectors were largely based on transcriptomics and

Figure 1



Predicted three-dimensional models of powdery mildew RNase-like effectors. Predicted three-dimensional protein folds are based on the crystal structure of the *Aspergillus phoenicus* Ribonuclease T1 **(a)**. The wheat powdery mildew CSEP AVRPM2 **(b)**, and the barley powdery mildew CSEP AVR_{A13} **(c)** are depicted. AVRPM2 and AVR_{A13} are encoded within the same effector gene family, and they are recognized by the evolutionary unrelated wheat PM2 and the barley MLA13 immune receptors, respectively.

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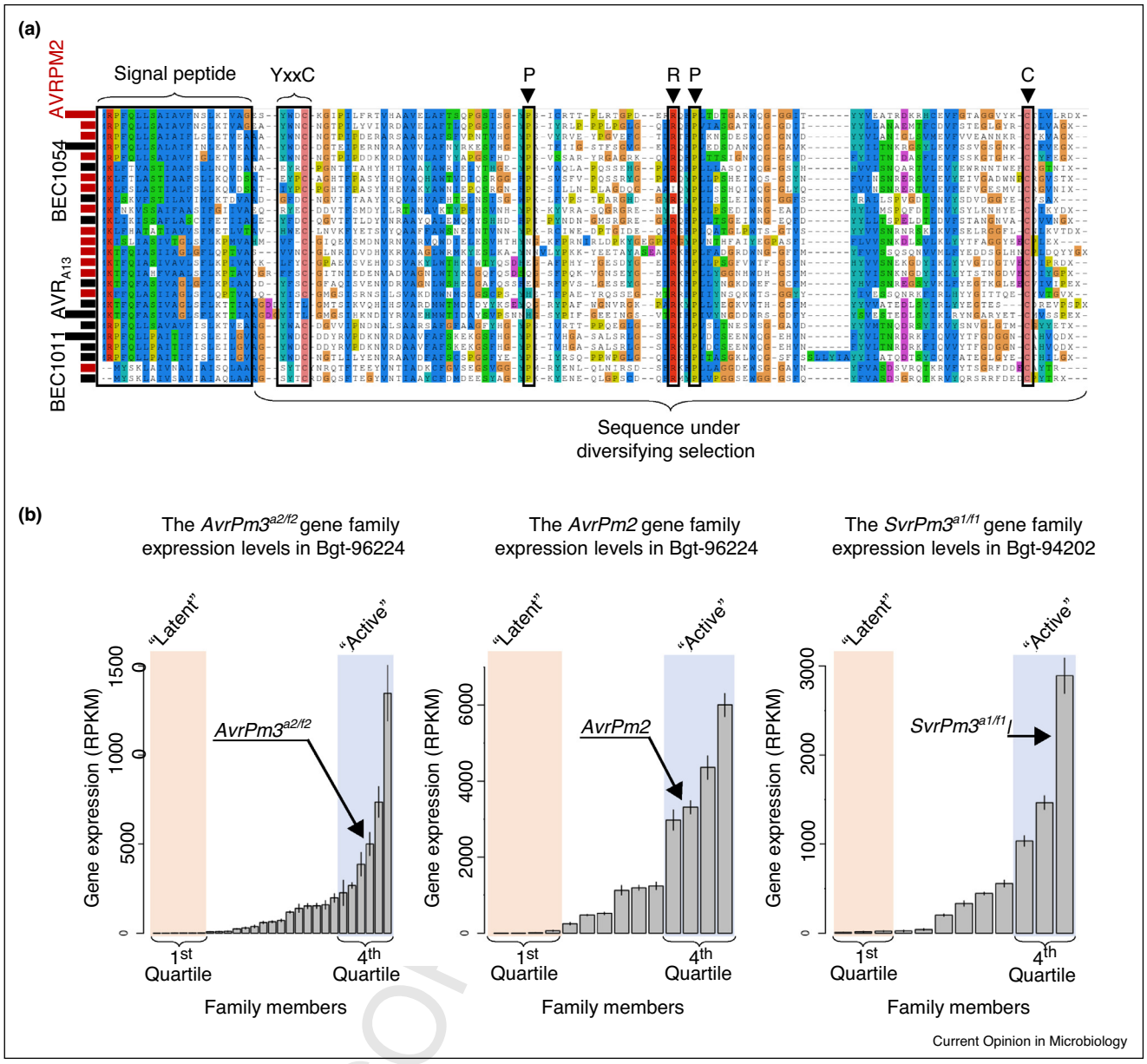
Figure 2



Common features of cereal powdery mildew effector gene families. **(a)** Protein sequence alignment of the AVRPM2 effector family members from *B.g. tritici* (red bars) and *B.g. hordei* (black bars). Protein sequences corresponding to the mildew AVR proteins AVRPM2 and AVR$_{A13}$, and the HIGS validated mildew *bona fide* effectors BEC1054 and BEC1011, are highlighted with prominent horizontal bars. The N-terminal predicted signal peptide, the conserved YxxC motif, as well as the most conserved residues are framed and annotated accordingly. Region under diversifying selection pressure is highlighted. **(b)** RNA-sequencing based assessment of gene expression levels across the *B.g. tritici* members of the *AvrPm3*$^{a2/f2}$ (left panel), *AvrPm2* (middle panel) effector gene families in the wheat powdery mildew isolate Bgt-96224, and the *SvrPm3*$^{a1/f1}$ effector gene family in the wheat mildew isolate Bgt-94202. Expression levels are given in reads per kilobase per million mapped reads (RPKM). Every bar corresponds to the expression of one family member at the wheat mildew haustorial stage (48 hour post infection), during compatible interaction between *B.g. tritici* and the susceptible wheat cultivar 'Chinese Spring' (Coraline Praz, unpublished data). Effectors in the first quartile are transcriptionally inactive and constitute a proposed group of latent effectors. By contrast, effectors in the fourth quartile are always highly expressed and encode for active *Avr* and *Svr* genes whose position in the plots is indicated with an arrow.

proteomics approaches in *B.g. hordei* [8–10,13]. In particular, RNA-seq monitoring of barley powdery mildew transcripts during the early stages of host infection revealed a two-step mode of action: a first wave of CSEP transcripts accumulated during host cell entry (12 hour), and a second wave of transcripts accumulated at the stage of haustorium formation (24 hour) [23•]. Similarly, high induction of CSEPs at the haustorial stage (48 hour) is

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also observed in wheat powdery mildew (Figure 2b; Coraline Praz, unpublished data). These results substantiate the importance of candidate effectors for mildew virulence, and suggest there are different subsets of CSEPs fulfilling distinct biological functions depending on the developmental stages of the fungus during host colonization. Indeed, Host Induced Gene Silencing (HIGS) [24••] of 21 individual barley powdery mildew effector genes resulted in significant reduction of host penetration and haustorium formation, further supporting the essential contribution of mildew CSEPs to the establishment of host infection [11,25•,26•,27]. Pliego and colleagues also showed that HIGS downregulation of 50 haustorially expressed barley mildew effectors resulted in highly variable effects on fungal virulence, ranging from a significant increase to a significant reduction of haustorium formation depending on the targeted effector gene [11]. This data suggests that some CSEPs are probably dispensable, while others such as BEC1054 and BEC1011, whose HIGS downregulation resulted in 60–70% reduction of haustorium formation, are acting as bona fide (i.e. true) effectors that are essential for mildew virulence [11].

There is evidence that mildew effectors interfere with components of host basal metabolism and host immunity, with prominent examples being CSEP0055 [25•], BEC3, BEC4 [28•], CSEP0105, CSEP0162 [26•], and BEC1054 [29•] from *B.g. hordei*, and *SvrPm3*$^{a1/f1}$ [19••,20] from *B.g. tritici* (Figure 3). Ahmed and colleagues showed that the sequence unrelated CSEP0105 and CSEP0162 both interact with the stress related small heat shock protein chaperones HSP16.9 and HSP17.5 from barley [26•]. We propose a possible mode of action of mildew CSEPs based on functional redundancy among effectors (Figure 3). In an approach combining pull-down assays from barley protein extracts and experimental validation by yeast-2-hybrid, Pennington and colleagues showed that BEC1054 physically interacts with a barley pathogen-related-5 (PR5) protein plus three sequence unrelated proteins fulfilling distinctly different biochemical functions, including a glutathione-S-transferase (GST), a malate dehydrogenase (MDH), and an elongation factor 1 gamma protein (eEF1G) [29•]. It is unclear if these interactions are based on the presence a conserved protein motif in all four targets serving as a binding site for BEC1054, or if the effector protein itself carries several protein–protein interaction domains allowing specific binding to multiple targets. It is also unclear how interactions between BEC1054 and its barley targets are affecting the host's metabolism or immunity. Nevertheless, the experimental data from Pennington and colleagues suggests another possible mode of action of mildew CSEPs based on specific binding to several host targets. Here, the action of a single effector can possibly disturb several biochemically distinct proteins involved in distinctly different pathways, which we could refer to as 'Effector disturbance' (Figure 3). Finally, in wheat powdery mildew, Bourras and colleagues provided genetic evidence that the SVRPM3$^{A1/F1}$ suppressor of AVR (SVR) recognition, is a suppressor of the wheat PM3A and PM3F-mediated race-specific resistance of wheat, which has been functionally demonstrated in *Agrobacterium*-mediated transient expression assays in *Nicotiana benthamiana* [19••]. There are only a few examples of SVR effectors from plant pathogens, with two other prominent examples being AVR1 from the tomato pathogen *Fusarium oxysporum* [30] and AVRLM4-7 from the oilseed rape pathogen *Leptosphaeria maculans* [31]. The mode of action of the SVRPM3$^{A1/F1}$ RNase-like CSEP is unknown, and we propose this can be based either on a ribonuclease pseudoenzyme activity [32] or on an effector disturbance function targeting components of the PM3 resistance signaling.

There are 167 family clusters that have been identified across *B.g. tritici*, *B.g. hordei*, *B.g. on Lolium*, *B.g. avenae*, and *B.g. poae* [7], which may possibly encode a large diversity of biochemical functions with different modes of actions. There are also significant differences at the gene expression level within families, with some members showing very high levels of expression at the haustorial stage, while others seem to be transcriptionally extinct (Figure 2b; Coraline Praz, unpublished data). One possible scenario is that 'active' effectors are exposed to selective pressure from the host, and are at risk of being recognized by the plant immune system. In such cases, these effectors are transcriptionally suppressed and become 'latent'. If this were the case, they could represent a reservoir of effectors which can be reactivated to compensate for the loss of function, mutation, deletion, or downregulation of other active effectors imposed by selective pressure to escape host recognition. We speculate that this transcriptional plasticity allows the preservation of high levels of genetic diversity in effector families to provide cereal mildews with a potential for cultivar and host adaptation. We also speculate that the very high repeat content of the mildew genomes might contribute to such plasticity by providing additional layers of regulation at the epigenetic level [33,34].

Mildew effectors recognized by the plant immune system

Genes encoding CSEPs in plant pathogenic fungi may act as avirulence genes (*Avr*) recognized by the plant resistance (*R*) gene based immunity. In the recent years, the identification of CSEPs encoding AVR proteins in mildews has been greatly accelerated by increasing integration of next-generation sequencing and high-throughput genotyping technologies. Mildew *Avrs* have been thus identified using map-based cloning [19••,22••], bulk-segregant-analysis (BSA) [19••,35], genome wide association studies (GWAS) [22••], and RNAseq based GWAS [21••]. This has allowed the cloning of four *Avr* effectors: the *B.g. tritici AvrPm3*$^{a2/f2}$, and *AvrPm2* which are recognized by

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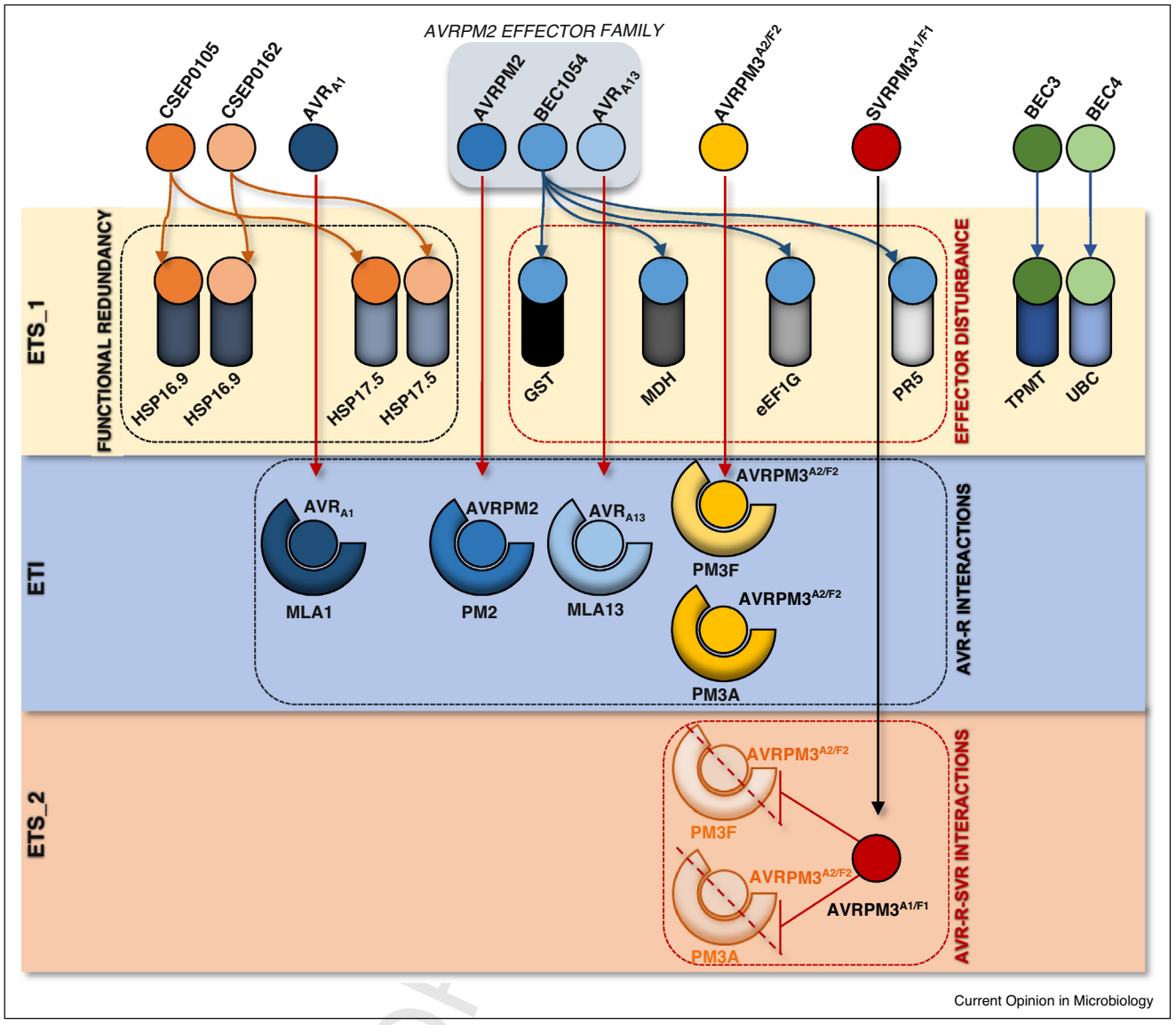
Figure 3



Contribution of cereal powdery mildew CSEPs to host–pathogen interactions. Three layers of plant–pathogen interactions commonly defined in the Zig-Zag evolutionary model are here represented. In this model, a subset of powdery mildew effector proteins (CSEP0105, CSEP0162, BEC1054, BEC3, and BEC4) are employed at suppressing the first layer of plant defenses commonly provided by PAMP-triggered immunity (PTI), or at manipulating the host cell to derive nutrients, thus resulting in a first layer of Effector-triggered susceptibility (ETS_1, upper panel). Here we highlight two possible modes of actions, namely 'Effector redundancy' and 'Effector disturbance' as well as functionally validated examples of effector host targets (see text for details). Another subset of effectors (AVR_{A1}, AVRPM2, AVR_{A13}, and $AVRPM3^{A2/F2}$), are probably also actively involved in virulence, but they are recognized by NLR receptors from the plant immune system (MLA1, PM2, MLA13, and PM3A/F, respectively), thus leading to Effector-triggered immunity (ETI, middle panel). These are basically canonical AVR–NLR interactions controlling race specific resistance in the host. Finally, a third subset of effectors ($SVRPM3^{A1/F1}$) are SVRs capable of suppressing such AVR–NLR mediated resistance thus providing a second layer of Effector-triggered susceptibility (ETS_2) based on the AVR-R-SVR model.

the wheat *Pm3a/f*, and *Pm2 R* genes, respectively [19••,22••], and the *B.g. hordei* Avr_{a1}, Avr_{a13} which are recognized by the barley *Mla1* and *Mla13 R* genes, respectively [21••]; and one suppressor of *Avr* recognition, $SvrPm3^{a1/f1}$ [19••,20]. *AvrPm2* and Avr_{a13} belong to the same effector gene family, and together with Avr_{a1} and $SvrPm3^{a1/f1}$ they encode for RNAse-like effectors (Figures 1 and 2a). One exception so far is $AvrPm3^{a2/f2}$, the cognate *Avr* of the *Pm3a* and *Pm3f* resistance gene alleles from wheat, which does not encode for a ribonuclease-like protein [19••]. Mildew *Avrs* are also members of size variable effector families, and all are among the most highly induced members upon infection (Figure 2b). This data suggests that members of the same

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effector gene family do not equally contribute to virulence, and that the most highly expressed ones are likely to be recognized by the plant immune system as avirulence factors.

Altogether, these results show that mildew effectors can act on the multiple layers of plant immunity described in the Zig-Zag model of plant–pathogen interactions [36,37] (Figure 3). Some effectors proteins such as BEC1054, may act as *bona fide* effectors suppressing PAMP-triggered immunity (PTI) and allowing successful host colonization through a first layer of effector-triggered susceptibility (ETS_1, Figure 3). The biochemical basis of such mode of action remains unclear since little is known about effector function in mildews. Other effectors such as AVRPM3$^{A2/F2}$, are canonical targets of plant immune receptors, whose recognition results in effector-triggered immunity (ETI, Figure 3). A recent study by McNally and colleagues revealed that *AvrPm3*$^{a2/f2}$ is highly conserved in worldwide wheat mildew populations, suggesting this CSEP may possibly act as an important virulence factor on susceptible hosts [38]. Finally, another type of CSEPs such as SVRPM3$^{A1/F1}$ are *bona fide* effectors that may act as SVRs suppressing ETI, allowing the pathogen to escape *R* gene recognition, and therefore achieving a second layer of ETS (ETS_2, Figure 3).

Future work on cereal mildew effectors

There is no transformation protocol for cereal powdery mildew fungi, nor is there any method for cultivation of these fungi on artificial media. These constraints impose the use of alternative approaches for the characterization of powdery mildew effectors based on heterologous plant, fungal, and bacterial expression systems. So far, studies combining HIGS, and effector targets screens (e.g. yeast-2-hybrid and pull-down assays) have succeeded in the functional validation of the effector function of several CSEPs, and in subsequent identification of several of their host targets. Other important and parallel efforts have resulted in the identification of CSEPs encoding mildew AVRs; several AVR–NLR pairs have been functionally validated using transient expression assays in barley protoplasts, and *Agrobacterium*-mediated transient expression in barley and in the heterologous *N. bethamiana* host, three assays amendable to high-throughput screens.

On the host side, more than 60 powdery mildew resistance genes have been described in the wheat gene pool, many of them with different alleles [39–41]. Similarly, in barley there are many resistance genes known, the large *Mla* allelic series being the most prominent [42,43]. Therefore, we propose that the identification of the cognate CSEPs for many of these resistance proteins, combined with functional characterization of their effector function and virulence targets, will provide a unique biological opportunity to describe the network of interactions between cereal powdery mildews and their hosts. It should also be possible to identify AVR encoding CSEPs based on common features of *Avr* genes in mildews (e.g. they are sequence polymorphic and highly expressed members of their effector gene family), which can be mined in the large number of available mildew genomes and transcriptomes. We also propose that the resolution of the three-dimensional structure of representative members of large CSEP families, can be a powerful tool to dissect effector protein functions based on structure-informed mutagenesis screens. Finally, the field of *Avr* population genetics is largely underexplored in cereal mildews, and we suggest the study of *Avr* diversity at the population level will be another powerful tool for understanding the evolutionary forces driving host–pathogen co-evolution in this agronomically important pathosystem.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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This paper describes the barley powdery mildew draft reference genome, as well as the analysis and comparison with two powdery mildews pathogens of dicotyledonous plants. Evidence is provided for a massive retrotransposon proliferation in the mildew genomes, coupled with a drastic loss of genes encoding enzymes of primary and secondary metabolism, carbohydrate-active enzymes, and transporters.

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This paper describes the wheat powdery mildew draft reference genome, the resequencing of three additional isolates, and comparative analyses with the barley powdery mildew genome. This study provides evidence that the mildew genomes are mosaics of ancient haplogroups that existed before wheat domestication.

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